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Bacterial Cell Production from Hexadecane at High Temperatures

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On mineral medium with hexadecane as the sole carbon source, stable mixed bacterial enrichment cultures were obtained from soil inoculum at 25, 35, 45, 55, and 65 C. Cell yields (grams of dry cells per gram of hexadecane) were determined for each of the enrichment cultures grown at the temperature at which they were enriched, and also for the 55 and 65 C cultures grown at various temperatures. In all cases, cell yields decreased with increasing growth temperature. The highest yield obtained at 65 C was 0.26, and the lowest yield obtained at 25 or 35 C was 1.02. Slower growth was observed at higher temperatures.

In spite of the technological advantages of high-temperature growth, there appear to be no figures in the literature on bacterial cell yields obtainable from hydrocarbons at high temperatures, although there are reports of growth at temperatures up to 70 C (8, 10). There are yield figures from many laboratories (7) for lower temperatures. The work described here was undertaken to determine whether cell yields could be obtained above 45 C that were equal to those obtainable below this temperature.

MATERIALS AND METHODS

Media. The medium used in fermentations for yield determination contained (in grams per liter); hexadecane, 6.5; KH_2PO_4, 2.72; Na_2HPO_4, 5.68; $(NH_4)_2SO_4$, 5.28; $MgSO_4$, 0.1; $CaCl_2$, 0.05; and (in milligrams per liter); $Fe(SO_4)_3$, 0.6; $ZnSO_4 \cdot 7H_2O$, 0.2; $CuSO_4 \cdot 5H_2O$, 0.2; and $MnSO_4 \cdot H_2O$, 0.2. The *p*H was 7.05. The medium used for enrichment was similar, except that it contained half as much KH_2PO_4, and one-fourth as much Na_2HPO_4, $(NH_4)_2SO_4$, and hexadecane as the fermentation medium. Its *p*H was 6.5. Mineral agar plating medium was made by adding 1.8% Ionagar no. 2 (Oxoid) to fermentation medium. Hexadecane was supplied from a piece of filter paper, moistened with hexadecane, in the petri dish cover.

Enrichment procedure. Soil was used to inoculate 100 ml of enrichment medium (octadecane was sometimes used instead of hexadecane) in 500-ml Erlenmeyer flasks incubated at the desired temperature. The flasks either were incubated on rotary shakers in constant temperature rooms, or were provided with magnetic stirring bars and individual temperature control, effected by a thermistor sensor and a heater. The flasks were closed with rubber stoppers and connected by Tygon tubing (1.6-mm bore, 1.6-mm wall) either to mercury manometers or to pressure transducers arranged to give an output of 10 mv for a pressure change of 150 mm of Hg. Growth was routinely followed by observation of the pressure decrease in the flask, corrected for barometric changes. Flasks were re-gassed with air as often as required to prevent oxygen deficiency. When the hydrocarbon had been exhausted, 10 ml of flask contents was used to inoculate a new flask, and the incubation was repeated. This procedure was continued until a stable culture was obtained.

Fermentor. The fermentor used for determination of cell yield has been described previously (11). It has a working volume of 3 liters, is 15 cm in diameter, and contains a draft tube with an impellor at its lower end, which was operated at 1,070 rev/min. Aeration was not at a constant rate, but was controlled by a dissolved oxygen probe (2). Simple on-off control was used, arranged so that the dissolved oxygen partial pressure did not fall below 0.05 atm. The *p*H of the medium was controlled at 6.7 by automatic addition of KOH, and the incubation temperature was automatically controlled by heating or cooling as required. The fermentor was not operated aseptically and was inoculated with stable enrichment cultures that were grown without asepsis. Because stable, presumably symbiotic associations of organisms were desired, asepsis was avoided except in culture analysis by plating.

Inoculum. Inoculum was grown in 500-ml flasks by using the same procedure as for enrichment, except that the amount of hexadecane used in the medium was only 0.43 g per liter, and incubation was for 1 or 2 days. At 25 and 35 C, 5% (v/v) inoculum was used, and at the higher temperatures 10% was used.

Analyses. A Nesslerization procedure was used

for direct determination (without distillation) of ammonium ion in centrifuged samples of culture. The exponential growth rate was calculated from the rate of nitrogen uptake thus determined. Fermentations were considered completed when a sample, centrifuged and held in the cold room to solidify any residual hexadecane, showed no trace of hydrocarbon at its surface. The amount of hexadecane lost by evaporation was calculated from the vapor pressure of hexadecane and the total volume of air used for aeration. The loss was about 0.3 g at 65 C, about 0.15 g at 55 C, and 0.07 g at 45 C. After completion of the fermentation, cells were centrifuged, washed, and dried for 24 hr at 105 C for yield determination. Samples were ground and sent for C, H, N, and ash analyses to Micro-Tech Laboratories, Skokie, Ill.

RESULTS

Cultures. Stable enrichment cultures were readily obtained at 25, 35, 45, 55, and 65 C. In all cases the cultures consisted of more than one organism. The culture enriched at 55 C contained three organisms, the others two organisms. The organisms could be separated by plating the mixed culture on the plating medium described. Individual colonies, transferred to another plate, would not grow, but a mixture of colonies of all the types in the association would grow. No attempt was made to identify the cultures. A brief description follows.

Culture S-1, enriched at 25 C, contained (A) a nonmotile, gram-positive, rod-shaped organism, 1 μm by 3 to 7 μm in size, and (B) a nonmotile, gram-positive, spherical organism, becoming gram-variable at the end of the growth period. The cells were about 1 μm in diameter. Both organisms formed colorless colonies on mineral agar.

Culture S-2, enriched at 35 C, contained (A) a nonmotile, gram-positive, rod-shaped organism, 1 μm by 2.5 to 4 μm in size, forming colorless colonies on mineral agar, and (B) a gram-positive, spherical organism identical with or closely resembling organism B of culture S-1.

Culture S-3, enriched at 45 C, contained two nonmotile, gram-positive, rod-shaped organisms. One (A) measured 2 to 6 μm by 1 to 1.5 μm, and the other (B) measured 1.5 to 2.5 by 1 μm. Both organisms formed yellow-to-pink colonies on mineral agar.

Culture S-4, enriched at 55 C, contained three organisms: (A) a nonmotile, gram-positive, rod-shaped organism measuring 4 to 6 μm by 1 to 1.5 μm, (B) a short, nonmotile, gram-positive to gram-variable, rod-shaped organism, 1.5 to 2.5 μm by 0.5 to 1 μm in size, and (C) a motile, gram-negative, rod-shaped organism measuring 2 to 4 μm by 0.5 μm. All three organisms gave colorless agar colonies, but centrifuged liquid cultures showed a pink lower layer and a white upper layer of organisms.

Culture S-5, enriched at 65 C, contained (A) a gram-positive, nonmotile, rod-shaped organism measuring 5 to 8 μm by 0.5 to 1 μm, and (C) a gram-negative to gram-variable, rod-shaped organism of questionable motility, measuring 1.5 to 2.5 μm by 0.5 to 1 μm. Both organisms gave colorless colonies. A third organism (B), closely resembling organism B of cultures S-1 and S-2, appeared in the culture when it was grown at low temperatures (see Table 3).

In the above list, organisms called "A" are nonmotile, gram-positive rods, organisms called "B" are gram-positive to gram-variable cocci or short rods, and organisms called "C" are gram-negative, motile rods.

Cell yield and composition. When each of the five mixed cultures was grown in a fermentor at the temperature at which it had been enriched, the results shown in Table 1 were obtained. The cell yields obtained at 25, 35, and 45 C are similar to those previously obtained in our laboratory for bacteria grown on alkanes (12), but the growth rates are somewhat higher. At higher temperatures, however, yields were lower and growth was slower. The composition of the cells (Table 1) appears to be similar to that previously found for bacteria grown on methane (11). The ratios in which the individual organisms of the mixtures were present were stable (Table 1).

Growth of one culture at various temperatures. When attempts were made to grow each of the five cultures of Table 1 at various temperatures, it was found that no culture would grow well at a temperature much above that at which it was enriched. At temperatures below the enrichment temperature, however, growth was good. When culture S-4 (enriched at 55 C) was grown at various temperatures, the results shown in Table 2 were obtained. It will be noted that changes in growth temperature changed the ratio in which the three organisms in the mixture occurred. At the lower temperatures, organism C completely disappeared. The cell yields and growth rates obtained at each temperature resemble those of Table 1, yield and growth rate both decreasing at higher temperatures.

Table 3 shows results obtained when culture S-5, enriched at 65 C, was grown at various temperatures. Here, again, the ratio in which the organisms occurred changed with temperature. The organism disappearing at low temperatures was, with both culture S-4 and culture S-5, a gram-negative rod. With culture S-

TABLE 1. *Cell yields and cell composition for cultures grown at the temperature at which they were enriched*

Culture	Temp of enrichment and growth (C)	Cell yield[a]	Doubling time[b] (hr)	Cell composition (%)				Approximate population ratio[c]		
				C	H	N	Ash	Organism A	Organism B	Organism C
S-1	25	1.12	1.1	49.2	7.1	11.9	6.3	40	60	0
S-2	35	1.10	1.0	49.8	7.2	9.9	6.4	50	50	0
S-3	45	1.00	1.2	49.6	7.1	9.8	6.4	40	60	0
S-4	55	0.49	2.5	45.9	7.2	8.8	6.6	40	40	20
S-5	65	0.26	4.0	46.5	7.1	10.6[d]	6.5	70	0	30

[a] Grams of dry cells per gram of hexadecane used. Mean of three experiments.
[b] During the period of most rapid growth.
[c] The individual organisms are described in the text.
[d] A first analysis for this sample gave 12.9% N.

TABLE 2. *Cell yields for culture S-4 grown at various temperatures*

Growth temperature (C)	Cell yield[a]	Doubling time[b] (hr)	Approximate population ratio[c]		
			Organism A	Organism B	Organism C
25	1.02	1.0	30	70	0
35	1.02	1.0	50	50	0
45	0.93	1.2	35	60	5
55	0.43	3.0	40	40	20
65	0.15	5.5	30	40	30

[a] Grams of dry cells per gram of hexadecane used. Single experiments at 25 and 35 C; others mean of duplicates.
[b] During the period of most rapid growth.
[c] Characteristics of organisms A, B, and C are given in the text.

5, however, which originally contained only two organisms, the place of the disappearing organism was taken by a gram-positive rod. Again, as in Tables 1 and 2, low growth rates and low cell yields were obtained at high temperatures. Cell composition was obtained for the organisms grown at the higher temperatures. No significant differences from the compositions of Table 1 are apparent.

DISCUSSION

The pattern of cell yield and growth rate is very similar in Tables 1, 2, and 3. Yields at lower temperatures were higher than those at high temperature, regardless of the temperature at which the culture had been selected. Cultures selected at low temperatures did not grow at high temperatures, while cultures selected at high temperatures performed better at temperatures lower than the selection temperature. While the results do not indicate that high-yielding, high-temperature cultures do not exist, they did indicate that such cultures might be difficult to find. Growth at 55 and 65 C was invariably slow. If there had been present, in the soil samples used, organisms or groups of organisms capable of rapid growth at high temperatures, they would very probably have been selected by the enrichment procedure used.

Mesophilic bacteria typically have a high negative temperature coefficient of growth rate above their optimum temperature (6). With thermophiles, this negative temperature coefficient is usually smaller (1, 4). Very low negative temperature coefficients for growth have been observed for thermophiles capable of utilizing alkanes by Mateles et al. (10) and in the work reported here. The high negative temperature coefficients observed for mesophiles are probably an indication that the growth-limiting process is thermal destruction of a protein (9). The lower coefficients often observed with thermophiles may well be a reflection of the balance between destruction and repair or resynthesis of cell metabolites or components.

Very often, bacteria require growth factors at high temperatures that are not required at low temperatures (1, 6). The thermophilic bacterium grown on alkanes by Mateles et al. (10) required a vitamin-supplemented medium. The thermophile isolated on tetradecane by Klug and Markovetz (8) grew only very slowly in the absence of growth factors. It is probable that the repeated isolation, in the present work, of stable mixed cultures is due to the poor growth of single thermophiles on media devoid of organic growth factors.

Thermophiles are found to grow readily at temperatures where many cell constituents are labile (1) and where biosynthetic processes are faulty. It would not be surprising if growth efficiency were lower at high temperatures, because of the necessity for continual resynthesis and repair. Forrest (4) found that, when *Zymomonas mobilis* was grown at temperatures

TABLE 3. *Cell yields and cell composition for culture S-5 grown at various temperatures*

Growth temperature (C)	Cell yield[a]	Doubling time[b] (hr)	Cell composition (%)				Approximate population ratio[c]		
			C	H	N	Ash	Organism A	Organism B	Organism C
25	1.06	1.0					30	70	0
35	1.08	1.0					50	50	0
45	1.06	1.5	46.8	7.0	8.9	6.8	60	40	0
55	0.36	3.0	46.4	7.3	9.4	7.6[d]	90	0	10
65	0.23	4.5	47.0	7.1	8.8	7.9	70	0	30

[a] Grams of dry cells per gram of hexadecane used. Single experiments at 25 and 35 C; others mean of duplicates.

[b] During the period of most rapid growth.

[c] Characteristics of organisms A, B, and C are given in the text. Organism B was not present in the original enrichment, but appeared when it was grown at lower temperatures. Under these conditions, organism C disappeared.

[d] A first analysis for this sample gave 3.8%.

above the optimum, cell yield was decreased. It is possible that low cell yields are typical for thermophilic growth. There are, however, very few data on the point.

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